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ORIGINAL ARTICLE

Hui Wei · Anik L. Dhanaraj · Lisa J. Rowland · Yan Fu
Stephen L. Krebs · Rajeev Arora

Comparative analysis of expressed sequence tags from cold-acclimated and non-acclimated leaves of *Rhododendron catawbiense* Michx

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Abstract An expressed sequence tag (EST) analysis approach was undertaken to identify major genes involved in cold acclimation of *Rhododendron*, a broad-leaf, woody evergreen species. Two cDNA libraries were constructed, one from winter-collected (cold-acclimated, CA; leaf freezing tolerance −53°C) leaves, and the other from summer-collected (non-acclimated, NA; leaf freezing tolerance −7°C) leaves of field-grown *Rhododendron catawbiense* plants. A total of 862 5′-end high-quality ESTs were generated by sequencing cDNA clones from the two libraries (423 from CA and 439 from NA library). Only about 6.3% of assembled unique transcripts were shared between the libraries, suggesting remarkable differences in gene expression between CA and NA leaves. Analysis of the relative frequency at which specific cDNAs were picked from each library indicated that four genes or gene families were highly abundant in the CA library including early light-induced proteins (ELIP), dehydrins/late embryogenesis abundant proteins (LEA), cytochrome P450, and beta-amylase. Similarly, seven genes or gene families were highly abundant in the NA library and included chlorophyll *a*/*b*-binding protein, NADH dehydrogenase subunit I, plastidic aldolase, and serine:glyoxylate aminotransferase, among others. Northern blot analyses for seven selected abundant genes confirmed their preferential expression in either CA or NA leaf tissues. Our results suggest that osmotic regulation, desiccation tolerance, photoinhibition tolerance, and photosynthesis adjustment are some of the key components of cold adaptation in *Rhododendron*.

Keywords Cold hardiness · Early light-induced protein · Expressed sequence tag · Genomics · Late embryogenesis abundant protein · *Rhododendron*

Abbreviations CA: Cold acclimated · ELIP: Early light-induced protein · EST: Expressed sequence tag · GAPDH: Glyceraldehyde-3-phosphate dehydrogenase · LEA: Late embryogenesis abundant protein · NA: Non-acclimated

H. Wei · A. L. Dhanaraj · R. Arora (✉)
Department of Horticulture, Iowa State University,
Ames, IA 50011, USA
E-mail: rarora@iastate.edu
Fax: +1-515-2940730

A. L. Dhanaraj · L. J. Rowland
USDA-ARS, Fruit Lab,
Beltsville, MD 20705, USA

Y. Fu
Interdepartmental Genetics Graduate Program,
Department of Genetics, and Developmental
and Cellular Biology, Iowa State University,
Ames, IA 50011, USA

S. L. Krebs
David G. Leach Research Station of the Holden Arboretum,
Mentor, OH 44094, USA

Introduction

Temperate zone woody perennials survive harsh winters through a process called cold acclimation, whereby plant tissues increase their freezing tolerance in response to shortening daylength, low non-freezing temperatures, and then sub-freezing temperatures, sequentially, through fall and winter (Weiser 1970; Sakai and Larcher 1987). In contrast, most herbaceous plants can be cold acclimated by exposure to a regime of moderately low but above freezing temperatures, and their cold acclimation capacity is typically much less than that in woody species (Kacperska-Palacz 1978). The development of cold acclimation is a complex process involving changes in gene expression that result in comprehensive alterations in cell walls, plant lipids, proteins, and carbohydrate metabolism (Guy 1990; Thomashow 1999), and the mechanisms for cold acclimation are inherently more complex in woody species than those in herbaceous plants (Ashworth and Wisniewski 1991; Welling et al. 1997; Rinne et al. 1998; Juntilla et al. 2002; Li et al. 2002; Karlson et al. 2003; Wisniewski et al. 2003).

cDNA microarray analysis of 8,000 genes in *Arabidopsis thaliana*, cold acclimated at 4°C for up to 1 week, indicated that 306 were cold-responsive, 70% being up-regulated, and 30% being down-regulated (Fowler and Thomashow 2002). These plants were subjected to somewhat reduced light intensity during cold acclimation as compared to control plants; however, the daylength was not changed (Fowler and Thomashow 2002). These results may have limited applicability to over-wintering woody species that respond to a combination of shortening daylength and sub-freezing temperatures through fall and winter and acquire maximal cold acclimation over a period of months (Weiser 1970; Sakai and Larcher 1987) as opposed to a few hours or few days as in herbaceous species (Chen and Li 1982; Ristic and Ashworth 1993).

We have been using broadleaf, evergreen members of the genus *Rhododendron* as a system for studying cold acclimation and freezing tolerance in woody plants (Lim et al. 1998b, 1999). These evergreen species and cultivars provide an opportunity to study cold acclimation biology in over-wintering leaf tissues without the interference of endodormancy transitions that occur in other tissues (buds) of deciduous woody plants (Lang 1987; Arora et al. 2003). Our group has recently conducted dehydrin profiling of cold acclimated and non-acclimated leaf tissues of 21 *Rhododendron* species (Marian et al. 2004), and showed that dehydrins—a family of proteins known to be induced during cellular dehydration—in the range of 25–73 kDa were more abundant in the cold acclimated than non-acclimated leaves of all the species, except in *R. brookeanum*, a tropical species that lacks cold acclimation ability. Furthermore, a close association between the levels of a 25 kDa dehydrin and the degree of leaf freezing tolerance was observed in both an F2 segregating population generated from a cross between a super-hardy and a hardy *Rhododendron* species (Lim et al. 1999) and in a comparative study of cold acclimated versus non-acclimated leaf tissues of six *Rhododendron* species with different freezing tolerances (Marian et al. 2004). However, a large-scale identification of changes in gene expression during cold acclimation in *Rhododendron* had not yet been attempted.

Expressed sequence tags (ESTs), which are generated by large-scale single-pass sequencing of randomly picked cDNA clones, have proven to be an efficient and rapid means to identify novel genes (and proteins) induced by environmental changes or stresses. These ESTs can be compared with EST, gene and protein databases and putative functions can be assigned to respective cDNAs. This information can then be used to gain insight into complex, mechanistic pathways of various stress tolerances. For example, this approach has been used to study gene expression in response to aluminum stress in rye (Milla et al. 2002), to water-deficit in maritime pine (Dubos and Plomion 2003), during leaf senescence in aspen (Bhalerao et al. 2003), and during cold acclimation in flower buds of blueberry (Dhanaraj et al. 2004).

We have initiated a project to construct an EST marker-based linkage map of *Rhododendron*, with a long-term goal to determine the location of dehydrins and other cold-induced genes as well as leaf freezing tolerance QTL on this map, thus to better understand the physiological and genetic basis of cold acclimation in evergreen, woody plants. As a first step in this initiative, we have used the comparative EST approach to detect changes in gene expression associated with cold acclimation in *Rhododendron*. Our hypothesis is that the different temperature and daylength regimes experienced by the cold acclimated (CA) and non-acclimated (NA) leaves of field-grown *Rhododendron* will be reflected in differential/preferential expression of cold-responsive, light-responsive, and photosynthesis-related genes in the leaves from the two treatments. Two cDNA libraries were constructed from CA and NA leaf tissues, and in total, 423 5′ ESTs were generated from the CA library and 439 5′ ESTs from the NA library. Here, we describe strategies to generate these EST datasets and analyze them to detect changes in gene expression during cold acclimation in *Rhododendron*. Northern blot analyses of selected genes, found to be preferentially abundant in CA or NA leaf tissues, were also performed to confirm their differential expression patterns.

Materials and methods

Plant material, cold acclimation treatment and tissue collection

Leaves were collected from field-grown plants of *Rhododendron catawbiense* 'Catalgla', a super cold-hardy genotype, at David G. Leach Research Station of The Holden Arboretum, Madison, OH, USA. The NA leaves were sampled during summer (July 2002); their leaf freezing tolerance, defined as the temperature causing the maximal rate of injury estimated by electrolyte leakage from leaf tissues in a freeze–thaw test, was measured to be −7°C using the method described by Lim et al. (1998a). The CA leaves were sampled from the same individuals during winter (January 2003); their leaf freezing tolerance was −53°C. Leaves were immediately frozen in liquid nitrogen and shipped on dry ice to Iowa State University, where they were stored at −80°C until RNA preparation.

RNA extraction

Total RNA was extracted from leaves according to the modified hot-borate method of Wilkins and Smart (1996). The RNA concentration was determined by spectrophotometry, and its integrity was assessed by electrophoresis in 1% (w/v) formaldehydeagarose gels (Sambrook et al. 1989).

cDNA library construction

Total RNA from CA and NA leaves were provided to Amplicon Express (Pullman, WA, USA) for poly(A^+) RNA purification and cDNA library construction using the Uni-ZAP XR vector (Stratagene, La Jolla, CA, USA). The synthesized double strand cDNAs (with *Eco*RI restriction site on the 5′-end and *Xho*I on the 3′-end) were directionally cloned into *Eco*RI/*Xho*I pre-digested Uni-Zap XR vector to make the phagemid cDNA libraries. The estimated titer for the primary CA and NA cDNA libraries was 8.0×10^6 and 2.2×10^6 pfu/ml, respectively.

Single-pass nucleotide sequencing

To convert the phage libraries to the plasmid form, mass excision of aliquots of the primary phage libraries was conducted according to the procedure described by Stratagene. After in vivo mass excision, about 500 colonies were randomly selected and cultured for plasmid extraction from each of the CA and NA cDNA libraries. Plasmid DNA from each clone was purified and used as templates for single-pass 5′-end nucleotide sequencing by Amplicon Express. Sequencing reactions were run on ABI 377 DNA sequencers (Perkin-Elmer Applied Biosystems, Foster City, CA, USA) and analyzed using Sequencher 4.1 software (Genecodes, Ann Arbor, MI, USA).

Editing and clustering of ESTs to identify unique transcripts

Quality score files for all of the original sequences were generated using the Phred program (Ewing and Green 1998; Ewing et al. 1998; http://www.phrap.org) and then imported into the Lucy program (Chou and Holmes 2001) for trimming of vector and low-quality sequence regions (Lucy parameters used: size 9, error 0.01 0.01, bracket 30 0.01) . The average Phred score for each EST was set at 20 (1% error possibility for each base). In addition, sequences shorter than 100 bases were not included in the analysis.

After Lucy trimming, the two sets of ESTs generated from the CA and NA libraries were clustered separately, using CAP3 assembly program (Huang and Madan 1999) with the following parameters: overlap identity = 95%, overlap length = 40 bp, clip range = 50, overhang = 20% [-p 95 -o 40 -y 50]. A list of contigs and singletons as well as their sequences were produced by CAP3 program for each library, and each contig or singleton was assumed to represent a unique transcript.

BLASTX search: EST homology comparisons, gene identification, and classification

To assign putative functions to cDNAs, edited sequences of unique transcripts were used as queries to search PIR-NREF protein database (Protein Information Resource: Non-Redundant Reference; release version. 1.42; Wu et al. 2002) using Standalone BLASTX program. The protein database of PIR-NREF was downloaded from the database website (http://pir.georgetown.edu/pirwww/search/pirnref.shtml), and formatted for BLAST program use. The Standalone BLASTX program was downloaded in the executable package from NCBI's FTP website (ftp://ftp.ncbi.nlm.nih.gov/blast/executables/). The expectation value (e-value) cutoff for BLASTX was set at > 1e-4. The best three protein hits for each EST were parsed out using Perl script, and were used to assign putative identities to the EST.

Northern blot analysis

Total RNA was extracted from NA and CA leaves using the protocol described earlier. Equal amounts of total RNA (8 μg) from each treatment were loaded and separated on 1% (w/v) formaldehyde-agarose gels and photographed to confirm RNA quality and to verify equal sample loading. RNA was subsequently transferred onto Brightstar-Plus nylon membranes (Ambion, Austin, TX, USA) by capillary transfer using the NorthernMax blotting and hybridization kit (Ambion), and was immobilized to the membranes by UV cross-linking.

For the preparation of DNA probes, cDNA inserts of interest were amplified by PCR from corresponding plasmids using T7 forward and T3 reverse primers. PCR products were precipitated with ammonium acetate and quantified by separating on 2% (w/v) agarose gels with low DNA mass ladder (Invitrogen Life Technologies, Carlsbad, CA, USA). About 25 ng of each cDNA insert was ^{32}P-labeled to a specific activity of 1.0–2.0×10^{-8} cpm μg^{-1} by random priming using the Megaprime DNA labeling system (Amersham Pharmacia Biotech, Piscataway, NJ, USA).

Membranes, with immobilized RNA, were prehybridized with ULTRAhyb buffer from the NorthernMax kit for 30 min and hybridized with ^{32}P-labeled DNA probes overnight at 42°C. Subsequently, the membranes were washed twice (15 min, 42°C) with low stringency wash solution from the NorthernMax kit and then exposed to X-ray film with intensifying screens at −80°C for autoradiography.

Results and discussion

Experimental strategy

According to their annual life cycle and leaf senescence characteristics, plants can be classified as herbaceous annuals, or deciduous or evergreen woody perennials. While there has been extensive research conducted on the genetic basis of cold acclimation in herbaceous

plants like *Arabidopsis*, relatively few studies have targeted deciduous and evergreen woody plants of the temperate zone. In deciduous woody plants, cold acclimation is often superimposed with leaf senescence and endodormancy of vegetative and floral meristems (buds), and, thereby, involves the alteration in the expression of not only cold acclimation-responsive genes, but also leaf senescence-responsive and endodormancy-responsive genes. Consequently, it is rather difficult to dissect the mechanistic pathways specifically involving cold acclimation. In evergreen *Rhododendron* leaves, however, cold acclimation-responsive gene expression is not confounded by other phenological factors as in deciduous species (Marian et al. 2004).

In this study, cDNA libraries were constructed from the leaves of NA and CA field-grown *Rhododendron* plants that were exposed to natural variation in daylength and temperatures through an annual cycle.

Quality of EST sequences

About 500 cDNA colonies were randomly picked from each of the NA and CA libraries. Each clone was assigned an identification name, consistent with its 96-well plate number and position. All selected clones were 5′-end sequenced in a single-pass. The generated ESTs were evaluated using Phred program, and vector and low-quality sequences were trimmed (see Materials and methods).

A total of 423 and 439 high-quality 5′ ESTs were obtained from the CA and NA libraries, respectively (Table 1). The EST length ranged from 109 to 733 bp with an average of 570 bp in the CA library, and from 106 to 690 bp with an average of 542 bp in the NA library. Length–frequency distributions for ESTs from each library are presented in Fig. 1. Since 75% and 65% of ESTs were longer than 500 bp, from CA and NA libraries, respectively, both EST datasets were judged to be of "good" quality.

Table 1 Number of ESTs, EST clustering and annotation of unique transcripts from non-acclimated (*NA*) and cold acclimated (*CA*) cDNA libraries of *Rhododendron catawbiense* leaf tissues

	NA	CA
1. No. of 5′ ESTs	439	423
2. Clustering of ESTs		
No. of contigs	46	35
No. of singletons	288	305
Total no. of unique transcripts	334	340
3. Annotation of unique transcripts		
Similar to known function genes	231 (69%)[a]	209 (61%)
Similar to unknown function genes	83 (25%)	77 (23%)
No significant similarity (novel)	20 (6%)	54 (16%)

[a]Values in parentheses represent the proportion of annotated genes of the total number of unique transcripts in each library

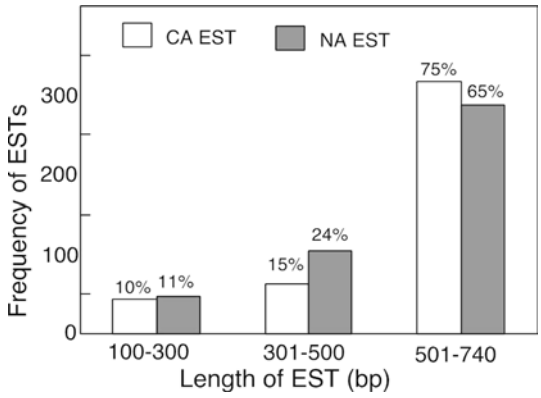

Fig. 1 The length–frequency distribution of ESTs from cold acclimated (*CA*) and non-acclimated (*NA*) cDNA libraries of *Rhododendron catawbiense* leaf tissues. The total number of ESTs was 423 and 439 from CA and NA libraries, respectively

Clustering analysis to generate unique transcripts

The two sets of ESTs were clustered using CAP3 assembly program (see Materials and methods). The clustering of ESTs from the CA library yielded 35 contigs and 305 singletons, with the number of unique transcripts being 340. For ESTs of NA library, 46 contigs and 288 singletons were generated, with the number of unique transcripts being 334 (Table 1, item 2).

To assess how many unique transcripts were shared between the CA and NA libraries, clustering analysis was also performed after combining all the ESTs from both libraries: a total of 646 transcripts (i.e. contigs and singletons) were formed; of those, 308 transcripts came from ESTs of CA library only, 297 from that of NA library only, and the remaining 41 included ESTs from both the libraries. Therefore, 6.3% (41/646) of the total unique transcripts were shared between the two libraries, suggesting remarkable differences in the pattern of gene expression between NA and CA leaves.

Dhanaraj et al. (2004) reported a similar percentage (4.3%) of the total unique transcripts being shared between the two datasets of about 600 ESTs from each of CA and NA libraries of blueberry floral buds, but stated that this could be an underestimation of the "actual" shared transcripts between the libraries. This is because sequencing from the 5′-ends can result in some sequences from cDNAs that represent the same transcripts not overlapping if all cDNAs are not full length. We made a comparison with another study (Bhalerao et al. 2003) which used >10 times larger EST datasets than that used in our study. Our analysis of their data indicated that only 9.1% (362/3988) of the total unique transcripts were shared between their two datasets, ~5,000 ESTs each from the cDNA libraries of summer (young) and autumn (senescing) leaves of aspen. Taken together, these results indicate that the levels of similarity between the datasets of ~500–5,000 ESTs from the control and stress-treated libraries were

in the range of 4.3–9.1%. These observations suggest that the datasets of ESTs obtained from CA and NA libraries in the present study are probably a reliable reflection of the whole library with respect to the abundant genes, thus providing a basis for a comparative analysis of gene expression under CA and NA conditions.

Putative identification and functional categorization of unique transcripts from CA and NA libraries

The sequences of unique transcripts (340 for CA and 334 for NA; Table 1, item 2), generated from the EST clustering, were translated into all possible reading frames and compared with the non-redundant protein database as described in Materials and methods. The annotation results were exported into Microsoft Excel data sheet and were re-sorted manually.

From the two libraries, 61–69% of unique transcripts showed similarity to proteins of known functions; 23–25% of unique transcripts showed similarity to proteins of unknown functions, and the remaining showed no similarity to any sequences in the databases and were deemed as "novel" (Table 1, item 3). It is worth noting that the "novel" transcripts constituted a higher percentage, 16% in CA library, as compared with 6% in the NA library (Table 1, item 3).

In order to keep the focus on nuclear genes, sequences representing chloroplast-encoded genes were manually removed from the analysis; in total, 20 ESTs (clustered into five transcripts) from NA cDNA library and four ESTs (clustered into three transcripts) from CA cDNA library were removed. Similar removal of such sequences has been previously reported for EST analyses of other plants (Ablett et al. 2000; Bhalerao et al. 2003).

The unique transcripts with known or predicted functions were classified into 14 functional groups and their subgroups (Table 2) according to the system used by Ablett et al. (2000) and Dhanaraj et al. (2004). The assignments into different categories were based on the authors' knowledge of biochemistry, plant physiology, and plant molecular biology, reference to the BioCyc-MetaCyc: Encyclopedia of Metabolic Pathways (http://www.MetaCyc.org/), and information contained in abstracts obtained from PubMed (http://www.ncbi.nlm.nih.gov/PubMed/).

Data from Table 2 indicate that the three functional groups with the highest percentage of cDNAs in NA library, in the descending order, were: Energy (category 02) > Primary metabolism (category 01) > Cell structure (category 09), while that in CA library were: Cell structure > Primary metabolism > Energy, suggesting there was a relative depletion in energy-related cDNAs but an enrichment in cell structure-related cDNAs during cold acclimation.

At subcategory level, the two classes with most significant enrichment of cDNAs during cold acclimation were "Glycolysis" and "Stress responses" (subcategories

Table 2 Abundance of transcripts expressed as the percentage of ESTs in each functional category (*underlined*) and corresponding subcategories for the non-acclimated (*NA*) and cold acclimated (*CA*) cDNA libraries of *R. catawbiense* leaf tissues

Functional category	Percent in NA	Percent in CA[a] (↑ or ↓ over NA)
01 Primary metabolism	8.21	10.87
01.10 Amino acid	1.14	1.42
01.20 Nitrogen and sulphur	0.46	0.47
01.30 Nucleotides	0.91	0.71
01.40 Phosphate	0.23	0.47
01.50 Sugars and polysaccharides	3.42	4.96
01.60 Lipid and sterol	1.37	2.13
01.70 Cofactors	0.68	0.71
02 Energy	25.06	10.64 (↓ ↓)[b]
02.10 Glycolysis (cytosolic)	0.46	1.89 (↑)
02.20 TCA pathway	0.46	0.24
02.30 E-transport: mito/chloroplast	9.34	6.15
02.40 Calvin cycle	9.12	1.42 (↓)
02.50 Photorespiration	1.82	0.00 (↓)
02.60 Others	3.87	0.95 (↓)
03 Cell growth/division	1.82	2.36
03.10 DNA synth/replication	0.68	0.24
03.20 Recombination/repair	0.46	1.18
03.30 Cell cycle/cell division	0.68	0.95
04 Transcription	3.88	3.07
04.10 RNA synthesis	0.46	0.24
04.20 General TFs	1.59	1.89
04.30 Chromatin modification	0.46	0.47
04.40 Unkown DNA binding motif	0.46	0.47
04.50 mRNA processing	0.91	0.00 (↓)
05 Protein synthesis	4.79	3.55
05.10 Ribosomal proteins	3.64	2.13
05.20 Translation factors	0.46	1.18
05.30 Translation control	0.23	0.00
05.40 tRNA synthases	0.46	0.24
06 Protein destination and storage	5.24	4.26
06.10 Folding and stability	0.68	1.18
06.20 Modification	0.46	0.00
06.30 Proteolysis	4.10	3.07
07 Transporters	1.82	1.42
07.10 Ions	0.68	0.47
07.20 Sugars	0.23	0.24
07.30 Lipids	0.23	0.47
07.40 ABC-type	0.23	0.24
07.50 Others or general	0.46	0.00
08 Intracellular traffic	2.06	1.42
08.10 Nuclear	0.46	0.24
08.20 Mitochondrial	0.23	0.24
08.30 Vesicular	0.68	0.71
08.40 Peroxisomal	0.23	0.00
08.50 Others	0.46	0.24
09 Cell structure	6.83	12.29 (↑ ↑)[b]
09.10 Cell wall/cell membrane	0.46	0.47
09.20 Cytoskeleton	0.23	0.00
09.30 ER/Golgi	0.00	0.24
09.40 Chloroplast	6.15	11.11
09.50 Others	0.00	0.47
10 Signal transduction	2.73	4.26
10.10 Receptors	0.00	0.71 (↑)
10.20 Mediators	0.46	0.24
10.30 Kinases	1.37	1.89
10.40 Phosphatases	0.68	0.47
10.50 G proteins	0.23	0.95 (↑)
11 Disease/defense	4.78	8.04
11.10 Resistance genes	0.46	0.00
11.20 Defense-regulated	1.14	0.24 (↓)
11.30 Stress responses	1.37	6.15 (↑)
11.40 Detoxification/antioxidants	1.59	1.42

Table 2 (Contd.)

Functional category	Percent in NA	Percent in CA[a] (↑ or ↓ over NA)
11.50 Heavy metals	0.23	0.00
11.60 Others	0.00	0.24
12 Secondary metabolism	1.37	1.65
12.10 Phenylpropanoids/phenolics	0.91	0.95
12.20 Terpenoids	0.23	0.00
12.30 Amines	0.23	0.71 (↑)
13 Development	1.83	1.65
13.10 Leaf and stem	0.69	0.71
13.20 Fruit and flowers	0.23	0.24
13.30 General/others	0.91	0.71
14 Hormone	0.46	0.95
14.10 Metabolism	0.23	0.00
14.20 Induced	0.23	0.95
Unclassified		
Unknown function genes	25	23
No significant similarity (novel)	6	16

[a](↑) or (↓) indicates a more than threefold higher or lower percentage, respectively, of cDNAs in CA library compared to NA library. If there were no cDNAs of a particular subcategory picked in one library (0), then, to determine if there was a threefold difference, the percentage picked from the other library was compared to 0.24 or 0.23, which is the percentage if one cDNA had been picked from the CA (1/423) or NA (1/439) library, respectively (as an example see subcategory 02.50 or 10.10)

[b](↑ ↑) or (↓ ↓) indicates that there was ~twofold difference in the percentage of cDNAs belonging to a functional category as a whole (such as category 02 of "Energy", or category 09 of "Cell structure") between the two libraries

02.10 and 11.30), while the two classes with most significant depletion of cDNAs during cold acclimation were "Calvin cycle" and "Photorespiration" (subcategories 02.40 and 02.50). Consistent with the relative depletion of energy-related cDNAs in CA library, the percentages of cDNAs encoding electron transport proteins were also relatively lower in CA library (subcategory 02.30) (Table 2).

Different genes were highly abundant in CA or NA libraries

Genes were defined as highly abundant when four or more ESTs were assigned the same gene function in a specific library. cDNAs that were picked many more times from one library than from the other were deemed as potentially differentially expressed transcripts. To investigate more closely, the differences as well as similarity in gene expression between the two libraries, the most abundant ESTs from each library were categorized as shown in Table 3.

Besides the possible preferential expression of distinct genes in NA versus CA treatments, two major differences between the libraries were gleaned from the data in Table 3: (1) there was a preponderance of genes encoding photosynthesis-related proteins among those that were highly abundant in NA leaf tissues and (2) cDNAs encoding early light-induced protein (ELIP), late embryogenesis abundant (LEA) protein/dehydrin, beta-amylase and cytochrome P450 were highly abundant in CA leaf tissues.

The cDNA abundance of glyceraldehyde-3-phosphate dehydrogenase (GAPDH), a cytosol and plastid glycolytic enzyme, was similarly high in both libraries, four ESTs in each (Table 3), suggesting that it is perhaps one of the housekeeping genes, a view also supported by a recent analysis of tomato ESTs (Coker and Davies 2003). However, since several nuclear-encoded isoforms of GAPDH exist in plant cells, we conducted a close bioinformatic analysis of GAPDH ESTs via manual BLAST search against the *Arabidopsis* protein database. The distribution of different GAPDH transcripts between the NA and CA *Rhododendron* cDNA libraries is shown in Table 4. Two cDNAs encoding chloroplast GAPDH subunits A and B (*GapA* and *GapB*) were found in NA library while only one (*GapA*) was found in CA library. In contrast, more cDNAs for cytosolic

Table 3 Most abundant transcript types in non-acclimated (*NA*) and cold-acclimated (*CA*) cDNA libraries of *R. catawbiense* leaf tissues

Putative gene identification	EST no. in NA library	EST no. in CA library	Enrichment ratio (NA:CA)
1. Most abundant in NA library			
(1) Chlorophyll *a*/*b*-binding protein	26	6	4.3:1
(2) RuBisCO small subunit precursor	13	1	13:1
(3) NADH dehydrogenase subunit 1	13	3	4.3:1
(4) RuBisCO activase	11	1	11:1
(5) Plastidic fructose bisphosphate aldolase	8	1	8:1
(6) Plastocyanin, chloroplast precursor	5	0	5:0
(7) Serine:glyoxylate aminotransferase	5	0	5:0
2. Most abundant in CA library			
(1) Early light-induced protein (ELIP)	0	40	0:40
(2) Late embryogenesis abundant (LEA) protein/dehydrin	1	18	1:18
(3) Beta-amylase	0	5	0:5
(4) Cytochrome P450	3	5	1:2
3. Most abundant in both libraries			
Glyceraldehyde-3-phosphate dehydrogenase	4	4	1:1

The ratio of abundance in NA:CA is calculated as "EST number in NA:EST number in CA", assuming no significant difference in the total number of unique transcripts between NA and CA libraries, which was 334 and 340, respectively

Table 4 The distribution of chloroplastic and cytosolic glyceraldehyde-3-phosphate dehydrogenases (*GAPDH*) ESTs between non-acclimated (*NA*) and cold-acclimated (*CA*) cDNA libraries of *R. catawbiense* leaf tissues

Putative gene identification		*Arabidopsis* ortholog ID	cDNAs from *Rhododendron* libraries		
			NA	CA	Enrichment ratio NA:CA
Chloroplastic GAPDH	*GapA* (subunit A)	At3g26650	NA4C03; 1e-50[a]	CA5D12; 5e-15	1:1
	GapB (subunit B)	At1g42970	NA4D06; 1e-63		1:0
Cytosolic GAPDH	Non-reversible GAPDH	At2g24270	NA1A11; 6e-87		1:0
	GapC	At3g04120	NA4H03; 4e-31	CA4H12; CA1D02; CA3H06; 2e-99	1:3
Total clones					4:4

[a]The e-value for the similarity search of the *Rhododendron* cDNA clones against *Arabidopsis* orthologs

GAPDH [*GapC*; clones CA4H12, CA1D02, and CA3H06) were found in CA library than that in NA library (clone NA4H03), suggesting *GapC* is potentially up-regulated during cold acclimation. These observations seem consistent with a previous study (Velasco et al. 1994) which demonstrated that the mRNA level and enzyme activity of cytosolic GAPDH (*GapC*) were significantly increased during dehydration or abscisic acid (ABA) treatment in desiccation-tolerant plant *Craterostigma plantagineum*. Our EST data also seem consistent with observations by Yang et al. (1993) that whereas the *GapC* mRNA level increased in *Arabidopsis* after heat-shock or anaerobiosis, *GapA* and *GapB* transcript levels remained constant or decreased under the same stress treatments. Our data from Table 4, taken together with the results presented in Table 2 (subcategory 02.10) that threefold more cDNAs for cytosolic glycolytic enzymes were picked from CA library than from NA library, suggest that enhanced cytosolic glycolysis rate was one of the cellular responses of cold acclimation in *Rhododendron* leaves.

Northern blot analysis of differentially abundant genes in CA versus NA leaf tissues

Northern blots (Fig. 2) were performed to examine expression of certain transcripts that were identified as likely to be differentially expressed based on their enrichment in one of the cDNA libraries (Table 3). Seven genes were selected as probes on blots containing total RNA from NA and CA *Rhododendron* leaves; among them, three were most abundant genes in NA library, including RuBisCO small subunit precursor, plastidic fructose bisphosphate aldolase and serine:glyoxylate aminotransferase, and another four were most abundant genes in CA library, including ELIP, LEA Dc3-like protein, dehydrin, and beta-amylase.

Results indicated that the transcript abundance of RuBisCO small subunit precursor, plastidic fructose bisphosphate aldolase, and serine:glyoxylate aminotransferase was higher in NA leaves as compared to CA leaves (Fig. 2), supporting our expectation based on EST analysis of these genes. Transcript abundance of

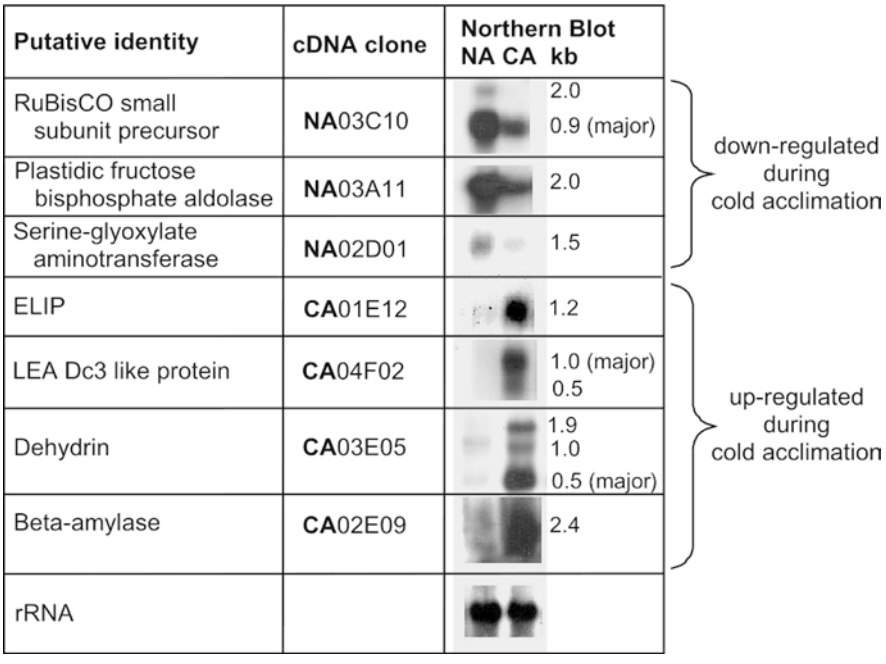

Fig. 2 Northern blot analysis of expression of transcripts identified as abundant in either the non-acclimated (*NA*) or cold-acclimated (*CA*) library of *R. catawbiense* leaf tissues. Total RNA was isolated from NA and CA leaves and hybridized with respective cDNA probes. *Lower panel* shows control hybridization of the filters to rRNA using a bluberry cDNA probe confirming equal loadings

ELIP, LEA Dc3-like protein, dehydrin, and beta-amylase was substantially lower in NA leaves compared to CA tissues (Fig. 2), which was also expected based on EST analysis of these two libraries. These results indicate that the approach to identify cold acclimation-responsive genes through the selection of highly abundant ESTs is a reliable one.

Chloroplast and peroxisome: photosynthesis and photorespiratory glycolate pathway genes were potentially down-regulated in CA leaves

Five distinct photosynthesis-related cDNAs were 4- to 11-fold higher in abundance in NA library than in CA library (Table 3); these genes encode the light-harvesting chlorophyll *a*/*b*-binding protein, the RuBisCO small subunit precursor, RuBisCO activase, plastidic fructose bisphosphate aldolase, and chloroplast precursor of plastocyanin, the latter required for the activity of photosystem I reaction center (Bengis and Nelson 1975). The low frequency of these cDNAs in the CA library suggests that the photosynthetic machinery is down-regulated in CA *Rhododendron* leaves. Our observations are consistent with that of a recent study by Jensen et al. (2003), who compared gene expression patterns in scions of the 'Gala' apple cultivar grafted to either M.7 EMLA or M.9 T337 rootstock. These authors noted that the photosynthetic genes were down-regulated in scions on M7 rootstock (compared to that on M9 where they were up-regulated) which were also more tolerant to various stresses, including low temperature.

Plastidic fructose bisphosphate aldolase is involved in the photosynthetic carbon reduction cycle. It catalyzes the synthesis of fructose bisphosphate from glyceraldehyde-3-phosphate and dihydroxyacetone phosphate, and is indeed down-regulated in CA *Rhododendron* leaf tissues (Fig. 2). Possible down-regulation of this gene was observed from EST analysis of autumn (senescing) leaf library (Bhalerao et al. 2003); however, it was not included in the reported inventory of cold-responsive genes in *Arabidopsis* (Fowler and Thomashow 2002), suggesting this gene is more likely to be regulated by changes in daylength rather than by low temperatures during cold acclimation. This interpretation is supported by the report that the promoter of this gene is light-regulated (Kagaya et al. 1995).

It is worth noting that a total of nine cDNAs encoding three key peroxisomal enzymes of the photorespiratory glycolate pathway were picked from NA library; among them, five for serine:glyoxylate aminotransferase (clones NA4G10, NA1B11, NA5H07, NA2D01, and NA5A09), three for glycolate oxidase (clones NA5C08, NA2G08, and NA5B10), and one for glutamate:glyoxylate aminotransferase (clone NA2F12), respectively. In contrast, no cDNA was found from CA library for these three photorespiration enzymes. Our Northern blot data (Fig. 2) confirm that serine:glyoxylate aminotransferase is indeed down-regulated in CA leaf tissues.

Photorespiration relies on the oxygenase activity of Rubisco and ribulose 1,5-P_2, a product of Calvin cycle (Siedow and Day 2000). The 2-phosphoglycolate produced by Rubisco's oxygenase activity is subsequently metabolized in the photorespiratory pathway, via the coordinated functions of the aforementioned three enzymes and other enzymes. Since ATP and reducing equivalents are consumed and CO_2 is released during this metabolic process, photorespiration is usually considered as a wasteful phenomenon. The likely down-regulation of photorespiration in CA *Rhododendron* leaves may be associated with the overall down-regulation of photosynthetic machinery, and could be beneficial for the plant to preserve the energy as well as the C reserve which are required for plant adaptation to cold temperature.

Mitochondria: genes involved in respiratory NADH oxidation were possibly down-regulated during cold acclimation

The cDNAs encoding NADH dehydrogenase subunit 1 were fourfold higher in NA library than in CA library (Table 3), with 13 ESTs identified in NA library and three ESTs in CA library; these ESTs can be clustered into one contig. As a component of proton pumping complex I in the respiratory chain, this enzyme is responsible for the oxidation of NADH and transfer of electrons to ubiquinone. The likely down-regulation of gene(s) encoding this enzyme in CA tissues is consistent with a recent study which demonstrated its down-regulation in cold stressed potato leaves (Svensson et al. 2002b). However, our results are in contrast with that observed for autumn (senescing) leaves of aspen, a woody deciduous plant, in that the cDNAs encoding NADH dehydrogenase subunit K were more abundant in the autumn leaf library than the summer one (Bhalerao et al. 2003). The NADH dehydrogenase has been previously identified as a senescence-associated gene (Buchanan-Wollaston 1997).

In intact mitochondria, complex I was found to be involved in the generation of activated oxygen species (Turrens and Boveris 1980). The possible down-regulation of genes for NADH dehydrogenase in CA *Rhododendron* leaves would potentially lower the risk for the formation of activated oxygen species, a well-known metabolic dysfunction associated with cold injury. The potential down-regulation of the genes associated with complexes I and the data in Table 2 (subcategory 2.30) indicating a lower percentage of cDNAs encoding electron transport proteins in CA library compared to NA library, suggest that the electron transport machinery may be sluggish in the CA *Rhododendron* leaves.

Early light-induced protein (ELIP) cDNAs were highly abundant in CA library: putative role in protection from photooxidation?

In this study, cDNAs encoding ELIPs were found to be the most abundant class in CA library, with 40 cDNAs picked from CA library as compared to none in NA library (Table 3). These ESTs were clustered into seven unique transcripts. Our Northern blot data, based on one of the ELIP transcripts, confirm that this gene is indeed up-regulated in CA leaves of *Rhododendron* (Fig. 2). ELIPs had also been found to be among the most highly induced genes in CA *Arabidopsis* leaves (Fowler and Thomashow 2002) and blueberry buds (Dhanaraj et al. 2004). However, their abundance in cDNA library of *Rhododendron* CA leaves was an order of magnitude higher than that in CA blueberry buds (40 vs. 4 ESTs in CA libraries and none in NA libraries).

Early light-induced proteins are nuclear-encoded thylakoid membrane proteins that were originally found to be transiently induced during greening of etiolated plants (Meyer and Kloppstech 1984). They accumulate within green tissues in response to a number of treatments including high light (Lindahl et al. 1997) and low temperature (Montane et al. 1997). They can bind chlorophyll *a* and leutein, and are speculated to function as photoprotective pigment carrier or chlorophyll exchange proteins to protect chloroplasts from light-induced damage (Adamska 1997). They might also provide protection against photooxidative damage through the dissipation of excessive light energy (Hutin et al. 2003).

Rhododendron plants are likely often exposed to a combination of freezing temperatures and high light in their natural habitat as under-story plants in deciduous forests. Possible down-regulation of the photosynthetic metabolism in over-wintering leaves, as noted in earlier sections, could potentially result in the light energy harvested by the leaves to be in excess of what can be processed by photosystems, thus making these plants particularly vulnerable to photoinhibition and photooxidative damage. In depth characterization of seasonal expression of ELIPs in *Rhododendron* leaves is warranted to further investigate their role in protection from photooxidation and winter survival of these plants.

Dehydrin/late embryogenesis abundant protein and beta amylase were highly abundant in CA library: cold and dehydration tolerance

Late embryogenesis abundant (LEA) proteins are a group of loosely related proteins that were originally discovered in higher plant seeds during late embryogenesis, and have been found to accumulate in plant tissues under various stresses such as drought, high salinity, and cold (Close 1996; Svensson et al. 2002a). In total, 18 LEA cDNAs were picked from CA cDNA library compared to only one from NA library in this study (Table 3); among them, eight belong to group 2 LEA subfamily and encode five distinct dehydrins (data not shown). Considering that in our previous studies (Lim et al. 1999; Marian et al. 2004), only one major dehydrin (~25 kDa) had been detected on Western blots of leaf proteins of *R. catawbiense* 'Catalgla' (from which the cDNA libraries were constructed), the EST analysis approach appears highly effective at identifying cold acclimation-responsive transcripts. Moreover, since cDNAs of dehydrins and other LEAs constitute the second most abundant gene family in the CA library, our results suggest a central role for these genes in cold acclimation, and support a microarray study with *Arabidopsis* which found LEAs to be the most abundant among the long-term up-regulated cold-responsive genes (Fowler and Thomashow 2002).

cDNAs encoding beta amylase were highly abundant in the CA library—five ESTs in CA and none in NA library. These results are consistent with earlier reports of its up-regulation in cold-acclimated *Arabidopsis* (Fowler and Thomashow 2002) and floral buds of blueberry (Dhanaraj et al. 2004). This enzyme catalyzes the breakdown of starch into simple carbohydrates, which could serve as osmoprotectants and, together with dehydrins, protect cells against freeze-induced desiccation stress. Our Northern blot data, based on representative transcripts for LEA, dehydrin and beta amylase, confirm that these genes are indeed up-regulated in CA leaves of *Rhododendron* (Fig. 2).

Conclusion

In this study, 862 *Rhododendron* ESTs were generated, providing sequences for primer design for marker development and genetic mapping of stress-related gene in *Rhododendron*, and, in addition, providing information on gene expression patterns of highly abundant transcripts involved in the cold acclimation process. From a functional categorization analysis of ESTs followed by Northern blot analysis, it appears that there is lower expression of genes involved in the Calvin cycle, photorespiration and electron transport during cold acclimation. In contrast, there appears to be higher expression of genes involved in glycolysis, cell structure and stress response during cold acclimation, suggesting that multiple cold-regulatory pathways may be involved. The most highly abundant cDNAs identified from the CA library include ELIPs, dehydrins/LEAs and beta amylase, all of which were confirmed to be up-regulated during cold acclimation and appear to be promising candidates for playing key roles in the development of cold tolerance in woody plants.

There are now several publicly available EST datasets of deciduous woody plants including poplar (Sterky et al. 1998), aspen (Bhalerao et al. 2003) and blueberry (Dhanaraj et al. 2004), of conifers such as pine (Allona et al. 1998; Whetten et al. 2001; Kirst et al. 2003) and sugi (Ujino-Ihara et al. 2000), and of other fruit tree

crops including apple (Sung et al. 1998), peach (Trainotti et al. 2003) and citrus (Hisada et al. 1997; Shimada et al. 2003). In addition, there is an ongoing EST project of birch (Tapio Palva, personal communication). The collection of high-quality ESTs generated in this study provides the first EST dataset for *Rhododendron*. The EST sequences have been deposited in GenBank at NCBI; the accession numbers for the ESTs of CA library are from CV014938 to CV015360 and for NA library are from CV015361 to CV015799.

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